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Rho GTPases: regulation and roles in cancer cell biology

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Abstract

Rho GTPases are well known for their roles in regulating cell migration, and also contribute to a variety of other cellular responses. They are subdivided into two groups: typical and atypical. The typical Rho family members, including RhoA, Rac1 and Cdc42, cycle between an active GTP-bound and inactive GDP-bound conformation, and are regulated by GEFs, GAPs and GDIs, whereas atypical Rho family members have amino acid substitutions that alter their ability to interact with GTP/GDP and hence are regulated by different mechanisms. Both typical and atypical Rho GTPases contribute to cancer progression. In a few cancers, RhoA or Rac1 are mutated, but in most cancers expression levels and/or activity of Rho GTPases is altered. Rho GTPase signaling could therefore be therapeutically targeted in cancer treatment.

Introduction

The Rho family of GTPases is part of the Ras superfamily. Rho GTPases are highly conserved and found in nearly all eukaryotes. They contribute to several cellular processes including organisation of the actin and microtubule cytoskeletons, regulation of gene expression, vesicle trafficking, cell cycle progression, cell morphogenesis, cell polarity and cell migration [1]. Furthermore, Rho GTPases also play an important role in pathological processes including cancer progression, inflammation and wound repair [2].

After cells are stimulated by extracellular factors such as soluble molecules, adhesive interactions or mechanical stresses, Rho GTPases can be activated and initiate signalling cascades through a wide range of effectors or targets including kinases and scaffold/adaptor-like proteins (Figure 1). The activation of Rho GTPases (GTP-bound form for the typical proteins) leads to changes in their conformation, increasing their ability to bind to effectors [3, 4].

The human Rho GTPase family consists of 20 proteins that are divided into 8 subfamilies, which are classified as typical or atypical depending on their mode of regulation (Figure 2) [5]. Rac, Rho, Cdc42 and RhoF/RhoD subfamilies are considered typical because they act as molecular switches, cycling between an active GTP-bound form and an inactive GDP-bound form. The ratio of GTP-bound form/GDP-bound form is regulated by guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs) and guanine nucleotide dissociation inhibitors (GDIs) (Figure 3). The atypical Rho GTPases are predominantly GTP-bound, and so far there is no evidence that they are regulated by GEFs or GAPs. However, like several typical proteins, there is good evidence that atypical proteins are regulated by other mechanisms, including expression and post-translational modifications, and that their function involves their additional domains that are not found in typical Rho proteins [6]. For

example, RhoBTB proteins have broad complex, tramtrack, bric à brac (BTB) domains that are able to interact with cullin3, a scaffold protein of ubiquitin ligase complexes involved in protein ubiquitination [7].

All Rho GTPases apart from the RhoBTB subfamily have been reported to alter cell morphology and/or the cytoskeleton when their expression is increased or reduced in cultured cells or model organisms. Here we describe how their activity is regulated, and their functions, particularly relating to cancer.

Regulation of typical Rho GTPases by GEFs, GAPs and GDIs

The three main types of proteins that regulate the activity of typical Rho GTPases in cells, GEFs, GAPs and GDIs (Figure 3), are often altered at the expression level in cancers, and some are mutated in a subset of cancers [8-11].

GEFs are proteins that accelerate release of bound GDP that is replaced with GTP, activating the GTPase. RhoGEFs are divided into two unrelated families: Dbl-homology (DH) domain family and Dock Homology Region (DHR) domain family. Most RhoGEFs are part of the DH family and they consist of a catalytic Dbl-homology (DH) domain followed by a pleckstrin homology (PH) domain. The DH domain catalyzes the exchange of GDP for GTP and the PH domain is believed to play a role in localising Dbl proteins to plasma membranes, and/or to affect the catalytic activity of the DH domain [9, 12]. GEFs of the DHR family have two conserved domains called DHR-1 and DHR-2. The DHR-2 domain interacts with the nucleotide-free form of Rho GTPases and forms an intermediate in the catalytic reaction for exchange of GDP to GTP [8, 13].

GTPase-activating factors (GAPs) are proteins that inactivate Rho GTPases by promoting the hydrolysis of GTP: they provide an essential catalytic group that accelerates the intrinsic GTPase activity of the GTPases. The human genome encodes around 80 RhoGAPs. However, less than half of them have been studied so far. RhoGAPs have a GAP domain of about 150 amino acids with a highly conserved arginine in a loop structure (arginine “finger”). This domain alone can bind to GTP-bound Rho proteins and catalyze their GTPase activity [14, 15].

Guanine nucleotide dissociation inhibitors (GDIs) control the cycling of some Rho GTPases between cytosol and membranes; and regulate the activation and inactivation of Rho GTPases. There are three RhoGDIs in humans: RhoGDI1-3. RhoGDIs consist of a N-terminal domain that interacts with the switch 1 and switch 2 domains of Rho GTPases, restricting the flexibility that is important for the GDP/GTP cycling; and a C-terminal domain that includes the geranylgeranyl-binding pocket that is important to extract geranylgeranylated Rho GTPases from the membrane [11, 16]. RhoGDIs inhibit the activity of Rho GTPases by extracting them from their sites of action in membranes, holding them in an

inactive form in the cytosol. On the other hand, they can act as chaperones to take Rho GTPases between membranes, and so may contribute to their activation in some cases [11, 17]. Finally, RhoGDIs have also been shown to protect some Rho GTPases from proteasomal degradation [18], presumably by binding to them and preventing their interaction with ubiquitin ligases.

Not all Rho GTPases interact with RhoGDIs [11, 19], and the interaction can also be regulated by post-translational modifications [20]. In addition, a Rac1 splice variant Rac1b has enhanced intrinsic guanine nucleotide exchange, impaired GTPase reaction and does not interact with RhoGDIs, leading to accumulation of the GTP-bound conformation of Rac1b in cells [21, 22].

Regulation of Rho GTPases by post-translational modifications

In addition to cycling between GTP- and GDP-bound conformations, Rho GTPases are regulated by post-translation modifications, including lipid modifications, phosphorylation,ubiquitination and SUMOylation (Figure 3).

Most Rho GTPases undergo post-translational lipid modification at their C-terminal CAAX motif (where C represents cysteine, A is an aliphatic amino acid, and X is a terminal amino acid). This motif is post-translationally prenylated on the cysteine, either by a farnesyl or geranylgeranyl isoprenoid lipid, followed by proteolytic removal of the three C-terminal (AAX) amino acids and methylation of the prenylated cysteine. These modifications are important for the translocation of Rho GTPases to the plasma membrane and/or endomembranes and are required for their biological activity [23]. Another modification that occurs near the C-terminus of several Rho GTPases is palmitoylation. For example, RhoU and RhoV do not have a functional CAAX motif, but instead have a C-terminal CFV motif that can be palmitoylated and target these proteins to membranes. A Cdc42 splice variant is a brain-specific isoform that can be palmitoylated (Cdc42-palm) instead of or in addition to being prenylated (Cdc42-prenyl). This change in lipid modification has an effect on protein localisation and function [24, 25].

Unlike all other Rho GTPases, the RhoBTB subfamily lack a CAAX motif and they are localized mainly in the cytoplasm [26]. So far there is no evidence that RhoBTB proteins are palmitoylated, but palmitoylation cannot be identified by amino acid sequence and requires biochemical analysis [27].

As well as lipid modifications, Rho GTPases undergo several other types of covalent modifications that can regulate the proteins in a positive (activation) or negative (inactivation/degradation) way (Figure 3).

Ubiquitination is the covalent attachment of an ubiquitin to lysine resides in the target protein. This modification often leads to protein degradation. However, it can also change

the localisation of proteins or their activity [28]. Ubiquitination of Rho GTPases can be triggered by bacterial toxins or growth factors [29], but other factors could be involved as well. Several Rho GTPases have been reported to undergo ubiquitination, including RhoA, Rac1, Rac1b, Cdc42, RhoB and RhoBTB2 [20, 30, 31]. Ubiquitination has been proposed to be a mechanism to control the local activity of Rho GTPases [30, 32, 33] and it can affect either the GTP-bound form (active) or GDP-bound form (inactive), or both. For example, Rac1 is ubiquitinated only when in the active form and bound to the plasma membrane [31, 34]. However, RhoA can be ubiquitinated in different conformations by different mechanisms. Nucleotide-free RhoA and GDP-RhoA are substrates of Smurf1 ubiquitin ligase [35] while only GDP-RhoA is a substrate of the BACURD-cullin3 complex [36]. Recently, it has been proposed that GDP-RhoA and GTP-RhoA are substrates of SCF^{FBXL19} E3 ubiquitin ligase, facilitated by Erk2-mediated phosphorylation of RhoA [37]. In all cases, the ubiquitination of RhoA leads to degradation by the proteasome.

SUMO is a ubiquitin-like protein that is similarly covalently linked to lysine residues. The only Rho GTPase that is known to be regulated by SUMOylation is Rac1, which becomes more active following addition of SUMO close to its C-terminus [38]

Phosphorylation is another covalent modification that regulates Rho GTPases. Some protein kinases such as PKA, ROCK1, Src and Akt have been shown to phosphorylate RhoA, Rnd3 (RhoE), Cdc42 and Rac1, respectively [20]. In most cases, phosphorylation seems to have a negative effect on the activity of Rho GTPases. RhoA can be phosphorylated on serine 188 (Ser188) by PKA and this allows RhoGDIs to bind with higher affinity to RhoA, thereby increasing its translocation from membranes to the cytosol. Although binding to RhoGDIs inhibits RhoA activity, the interaction can protect RhoA from ubiquitin-mediated degradation [39, 40]. Phosphorylation of Rnd3 by ROCK1 and PKC on 7 Ser/Thr residues increases protein stability and translocates Rnd3 to the cytosol which reduces its activity in inducing stress fibre disruption [19, 41]. Rac1 is phosphorylated on serine 71 (Ser71) by Akt and threonine 108 (Thr108) by ERK. Phosphorylation of Ser71 appears to decrease GTP-binding without affecting GTPase activity of Rac1 while phosphorylation of Thr108 inhibits interaction of Rac1 with PLCγ1 and induces translocation of Rac1 to the nucleus [42, 43].

Transcriptional and post-transcriptional regulation of Rho GTPases

Expression of the best-characterized 'classical' Rho GTPases, RhoA, Rac1 and Cdc42, does not vary much between different tissues. However, some Rho GTPases are restricted to particular tissues and in some cases, specific stimuli increase their expression or genotoxic stress [45,151]. Atypical Rho GTPases have also been shown to be regulated in a transcriptional level. For example, expression of *Rnd3* is induced after Raf activation [46], and expression of *RhoU* is increased by Wnt-1 and Notch1 [47, 48].

Rho GTPases undergo post-transcriptional regulation (Figure 3). microRNAs (miRNAs) are non-coding RNA molecules that can control the expression of mRNAs. These short sequences silence target genes by either inhibiting translation or degrading mRNA. Several Rho GTPases can be regulated by miRNAs. Most of the work on miRNAs has been done in cancer models, showing how the regulation of Rho GTPase expression by miRNAs can affect cancer progression [49]. For example, it has been shown that RhoA is target of miRNA-155 [50, 51] and miRNA-125a-3p [52]; RhoB is a target of miRNA-21 [53]; RhoBTB1 is a target of miRNA-31 [54]; and Cdc42 is a target of miRNA-29 [55] and miRNA-137 [56]. Sometimes the same miRNA can target two different Rho GTPases. miRNA-185 has been reported to decrease the levels of RhoA and Cdc42, leading to inhibition of proliferation in human colorectal cancer cells [57].

Rho GTPases and the actin cytoskeleton

A highly conserved function of Rho GTPases is the control of the actin cytoskeleton (Figure 1). Several cellular processes including cell migration, cell division, endocytosis and chemotaxis depend on the actin cytoskeleton [58], and it plays a central role in cancer cell migration and invasion [59].

Several Rho GTPases stimulate the first step of actin polymerization,known as nucleation, which involves the formation of a stable multimer of actin monomers that will function as a template to the elongation of the new filament. This is controlled by actin-nucleating proteins, including the actin-related protein 2/3 (Arp2/3) complex and formins. Each actin-nucleating protein acts in a distinctive mechanism. For example, Arp2/3 complex initiates a new actin filament that branches off an existing filament while formins promote nucleation of unbranched filaments at the barbed end of filaments [60, 61].

The Arp2/3 complex is not an efficient nucleator and it requires the activity of nucleation promoting factor (NPF) proteins, including Wiskott-Aldrich Syndrome protein (WASP), neuronal WASP (N-WASP) and WASP-family verprolin-homologous protein (WAVE; also known as suppressor of cyclic AMP repressor (SCAR)) [60, 62]. These proteins have in common a WCA domain, which consist of a WASP-homology-2 (WH2 or W); and a central (also called cofilin-homology or connector) and acidic (CA) region. These regions are important to bring G-actin to the complex and to change the conformation of the Arp2/3 complex that leads to the initiation of actin polymerization. NPFs are regulated by several Rho GTPases, for example Cdc42 activates N-WASP, and Rac1 activates a protein complex called the WAVE-regulatory complex (WRC), that includes WAVE [63-65].

As well as stimulating Arp2/3 complex-mediated actin nucleation, Rho GTPases bind to and activate formins (Figure 1), which are large multidomain proteins defined by the presence of

a catalytic formin homology 2 (FH2) domain. There are eight formin families identified in humans including Dia, FMNL and FHOD, which are targets of Rho GTPases [66]. Formins act both as nucleation and elongation factors. These proteins processively associate with barbed ends, allowing the addition of G-actin while inhibiting capping proteins from ending elongation [63, 66, 67].

Another family of proteins that control actin filament elongation is the Enabled/vasodilator-stimulated phosphoprotein (Ena/VASP) proteins. All members of the family have an amino-terminal Ena/VASP homology 1 (EVH1) domain followed by a proline-rich central region and a carboxy-terminal Ena/VASP homology 2 (EVH2) domain. They act by binding at barbed ends of actin filaments and inhibiting filament capping by capping proteins. Ena/VASP proteins elongate filaments that have been initiated by W-based filament nucleators such as an Arp2/3/NPF complex, and interact with the WRC to enhance Rac-mediated actin polymerization [68] Ena/VASP proteins can also control actin filament branching by reducing the density of Arp2/3-dependent actin filament branches [63, 68].

Filaments grow until they are capped. Capping is important to control the length of the growing branches and localise where the filaments generate propulsive forces. The two main proteins involved in this process are capping protein (also known as CapZ in muscle) and gelsolin [69]. While there are no direct interactions known between Rho GTPases and capping proteins, Rho GTPases regulate actin depolymerization, which is important for actin filament dynamics. The actin-depolymerizing factor (ADF)/cofilin family are involved in this step. These proteins are known to sever filaments and elevate the levels of monomeric actin. Both ADF and cofilin bind to ADP-bound subunits of the F-actin, promoting their disassembly. Cofilin can also bind to released ADP-actin monomers, controlling the recycling of disassociated actin subunits. ADF/cofilin activity is inhibited by LIMK-induced phosphorylation, and LIMK in turn is activated by the Rho effector ROCK and/or the Rac/Cdc42 effector PAK [58]. Hence Rho signaling not only increases actin polymerization but reduces depolymerization. The extent of depolymerization of the actin filaments relies on several factors including the relative concentration of other actin-binding proteins [70, 71].

Rho GTPases and cell migration

Cancer cells can use several different modes to migrate, and indeed can transition from one mode to another during cancer progression and metastasis [72]. Single cells are able to migrate in two interchangeable modes of migration: amoeboid and mesenchymal, while cells migrating as groups can use different protrusive structures at the front [73, 74]. Cell migration is a multistep process in which cells need to extend membrane protrusions in the cell front (lamellipodia, filopodia and/or membrane blebs), interact with the extracellular matrix or

neighbouring cells, contract the cell body and detach the cell rear from the surrounding environment (Figure 3). Rho GTPases are critical molecules in this process, sending signals from membrane receptors to the cytoskeleton and cell adhesions [75].

RhoA has been implicated in both lamellipodium-driven and bleb-driven migration in 3D environments (Figure 4). The bleb-driven mode of migration is characterized by actomyosin-based cortical contractility mediated by Rho/ROCK signalling. ROCK acts by increasing the phosphorylation of myosin light chain, which increases the interaction of myosin II with actin filaments [76]. The consistently high cortical tension results in blebbing, which contributes to cancer cell motility [76, 77]. Cells using lamellipodium-driven cell mnigration have an elongated morphology that depends on cell adhesion dynamics and traction forces between both poles of the cell. Lamellipodia drive protrusion of the plasma membrane at the front of the cell (Figure 4). Formation of integrin-based adhesions and actomyosin-mediated contractility is controlled by Rho/ROCK signalling [77, 78].

Lamellipodia are transient structures and their formation is driven by actin polymerization. The Arp2/3 complex is associated with actin filaments throughout the cell front, and together with WAVE proteins, it stimulates the formation of a “dendritic” actin network that is important for lamellipodium extension [79]. Activation of Rac is necessary for lamellipodium formation (Figure 1, Figure 4). GTP-Rac binds directly to Sra1 protein, activating the WAVE regulatory complex (WRC) (hetero-pentameric complex formed by Sra1/Cyfit1, Nap1/Hem2/Kette, Abi2, HSPC300/Brick1 and WAVE1/SCAR) [68, 80]. WRC activity is controlled by inhibition of the verprolin-homology, central and acidic (VCA) region. It is believed that the interaction of GTP-Rac with Sra1, and perhaps with the meander region of WAVE1 (a meandering path across a concave surface of Sra1 that is formed by five helices (α2-α6 and a series of intervening loops)), could lead to changes in the conformation of the complex and release of the VCA region, which is important for the activation of the Arp2/3 complex [81, 82].

The presence of $PI(3,4,5)P_3$ increases GTP-bound Rac in several cell types. It is thought that $PI(3,4,5)P_3$ promotes GTP loading on Rac through direct interaction with Rac GEFs. Several Rac GEFs, including Tiam-1, β-PIX and DOCK180, are known to activate Rac to induce lamellipodia. Rac activation also stimulates PI3K [83], which leads to the production of $PI(3,4,5)P_3$. This creates a positive feedback loop, accumulating active Rac at the cell front [75, 79, 84]. Another example of a mechanism to control actin polymerization in the cell front is through the PAK family of serine/threonine kinases. Activation of Rac and Cdc42 activates PAKs, which then phosphorylate and activate LIM kinase (LIMK). Activation of LIMK leads to phosphorylation and inactivation of cofilin. Cofilin is an important protein to promote filament treadmilling at the leading edge [70].

Formins are also involved in lamellipodium extension [60]. For example, it was found that the formin mDia1, a RhoA effector (Figure 1), localizes at the leading edge of some cells,

including T-cells, and it can cooperate with the Arp2/3 complex to initiate lamellipodium formation [85, 86]. RhoA is mainly active at the cell rear, but it has been shown to be active at the cell front as well (Figure 4). During membrane protrusion, RhoA is active at the leading edge, but it is inactivated during membrane retraction [87]. Activation of mDia1 by RhoA in the cell front could act together with the Arp2/3 complex to stimulate actin polymerization [85, 86].

Some cells use traction forces to pull on the extracellular matrix, which contributes to their migration. Active RhoA is associated with focal adhesion formation (sites of contact of the cell with the extracellular matrix) and cell contractility during cell migration. One important Rho effector that is involved in actin-myosin II filament assembly is the serine/threonine kinase ROCK. Activation of ROCK leads to phosphorylation and activation of LIMK, which then phosphorylates and inactivates cofilin. ROCK also phosphorylates myosin light chain (MLC) phosphatase, inactivating it. This leads to an increase in the levels of phosphorylated myosin light chain (pMLC), which then stimulates the cross-linking of actin filaments by myosin II and generates contractile forces. Contraction promotes membrane blebbing, movement of the cell body and contributes to detachment of the cell rear [84, 88]. Another important effector of RhoA is mDia. This effector cooperates with ROCK for the assembly of actomyosin bundles such as stress fibres. mDia is found at the front and back of the cell, and its function is dependent on the cell type and conditions during cell migration [84, 89].

Cell polarity is important for directional migration. Formation of protrusions and retraction of the cell rear are not enough to direct the cells to a specific place. The nucleus, Golgi apparatus and microtubule-organizing centre (MTOC) also need to be repositioned towards the leading edge [90]. Cdc42 has been shown to regulate MTOC positioning through recruitment of Par6 and aPKC to the leading edge [91]. Nuclear movement can also be regulated by Cdc42, probably through actin flow controlled by actin-myosin regulatory kinase (MRCK) [92-94].

Functions and regulation of atypical Rho GTPases

The atypical Rho GTPases have amino acid substitutions in their Rho domains that alter their GTP/GDP cycling. They consist of four subfamilies: RhoU/RhoV subfamily, RhoH subfamily, Rnd subfamily, and RhoBTB subfamily (Figure 1). Several of them, including Rnd3, RhoBTB2 and RhoH, have been implicated in cancer progression.

RhoU (also known as Wrch1) and RhoV (also known as Chp or Wrch2) proteins have significant sequence identity to Cdc42 (~52-55%), but they contain extra N- and C- terminal extensions that are critical for their activity [95, 96]. For example, the N-terminal domain is able to regulate RhoU and RhoV function in a negative way, a characteristic not reported for other Rho GTPases [96, 97]. Like Cdc42, RhoU and RhoV affect the actin cytoskeleton

(lamellipodium formation and filopodium formation, respectively), cell migration and formation of focal adhesions [6].

RhoU has a very rapid intrinsic guanine nucleotide exchange rate compared to Cdc42, and is believed to be mostly GTP-bound in cells [96]. RhoV is assumed to have similar properties, although this has not been tested biochemically. So far, RhoU and RhoV GAPs and GEFs have not been identified; although RhoU has been reported to bind to two GAPs, it is not yet known whether they act as GAPs for RhoU [98]. Expression of RhoU and RhoV is increased at the transcriptional level by Wnt-1; and RhoU has also been shown to be induced by STAT3 and Notch1 [47, 48, 99, 100]. The N- and C-terminal domains of RhoU and RhoV are quite different from each other. The N-terminal domain of RhoU but not RhoV contains proline-rich domains that bind to the SH3 domains of Grb2 and Nck [101], and its C-terminal domain is phosphorylated on tyrosine 254 by Src [96, 102]. Other known binding partners for RhoU and RhoV are the PAK family of kinases, which also interact with Cdc42 and Rac [47, 95, 103, 104].

RhoH, also known as TTF (translocation three four), is expressed predominantly in hematopoietic tissues, similarly to Rac2 [105]. RhoH is GTPase-deficient, and is therefore assumed to be constantly in a GTP-bound form. Due to the lack of intrinsic GTPase activity, RhoH is unlikely to be regulated by GAPs and GEFs. However, RhoH was reported to interact with RhoGDIs, although the functional consequence of this has not been investigated. It has also been shown to be regulated at the transcriptional level and by phosphorylation [106-108]. RhoH is important in the regulation of proliferation, survival, migration and engraftment of murine hematopoietic progenitor cells [109]; and it has been implicated in T-cell differentiation [110]. RhoH does not seem to have any direct effect on actin reorganization [26]. However, RhoH can antagonize the activity of other Rho GTPases. For example, Rac1 and RhoA-mediated activation of NFκB is inhibited by RhoH through inhibition of IκB degradation [108]. Loss of RhoH in murine hematopoietic progenitor cells leads to an increase in Rac1-mediated migration and cortical F-actin assembly [111], and RhoH deletion delays progression in a murine model for chronic lymphocytic leukemia [112, 113].

Like RhoH, Rnd1, Rnd2 and Rnd3 (also known as RhoE) proteins are GTPase deficient, and are therefore constitutively in the GTP-bound form [114]. One important function of Rnd1 and Rnd3 is that they can antagonize RhoA activity by interacting with p190RhoGAP. This interaction leads to loss of stress fibres and cell rounding [115, 116]. Other proteins interact with Rnd proteins, which could also contribute to Rnd-induced responses [114].

Rnd1 and Rnd3 are mainly associated with membranes and RhoGDIs do not affect their localisation. Rnd2 seems to be localised predominantly in the cytoplasm [23, 114]. Since Rnd proteins have not been shown to interact with any GAPs or GEFs, these proteins should be regulated by other mechanisms such as phosphorylation, expression and localisation. In fact, it has been shown that Rnd proteins can be phosphorylated and phosphorylation (in

combination with farnesylation) allows these proteins to interact with 14-3-3 proteins. This interaction leads to translocation of Rnd proteins from the plasma membrane to the cytosol, inactivating these proteins [19]. Rnd3 is known to be phosphorylated on 5 serines and 1 threonine by ROCK1 and on serine 210 by PKC [19, 41, 117]. Moreover, expression of Rnd3 is induced by activation of Raf, leading to changes in the actin cytoskeleton [46].

In mammals, RhoBTB1 and RhoBTB2 form the RhoBTB subfamily of Rho GTPases. Orthologues of RhoBTB1 and RhoBTB2 are present in vertebrates and insects, but not in plants and fungi [118, 119]. Although RhoBTB3 shares some similarities with RhoBTB1 and RhoBTB2, this protein is not considered a Rho GTPase due to the low similarity of its Rho domain to other Rho proteins [44, 118]. The Rho domain of RhoBTB1 and RhoBTB2 contains the amino acid motifs required for GTP-binding but is around 18 residues longer and rich in charged residues [120, 121]. The GTP-binding domain of RhoBTBs is expected not to hydrolyse GTP, because they do not have key amino acids required for GTP hydrolysis, including a glycine equivalent to G12 and a glutamine equivalent to Q61 in Ras [121, 122]. It has been reported that the GTP-binding domain of RhoBTB2 binds to GTP *in vitro* [120], but nothing has been published for RhoBTB1. RhoBTB1 and RhoBTB2 are also bigger than classical GTPases due to the presence of extra domains including 2 broad-complex, tramtrack, bric à brac (BTB) domains, which are conserved protein-protein domains. It has been shown so far that the BTB domains in RhoBTB proteins are important for the formation of Cullin-RING ubiquitin ligases (CRLs), the most prevalent class of E3 ubiquitin ligases [7, 123]. Other BTB-containing proteins interact with cullin3, acting as adaptors that target proteins to the Cullin3-RING ubiquitin ligase complex (CRL3) for ubiquitination and hence degradation [124]. Indeed, RhoBTB2 has been shown to be a substrate for the cullin3 ubiquitin ligase complex [123].

Relatively little is known about the functions of RhoBTB proteins. RhoBTB2 is involved in transporting vesicular stomatitis virus glycoprotein (VSVG) in a microtubule-dependent manner [125]. It has been reported that RhoBTB2 has an active role in cell cycle progression and apoptosis through the transcription factor E2F1 [126]. Moreover, RNA profiling using Hela cells has shown that RhoBTB2 affects cell cycle, apoptosis, cytoskeleton and membrane trafficking pathways [127]. RhoBTB1 and RhoBTB2 were observed to localize to membrane vesicles, but the functional relevance of this is not known [26].

Rho GTPases in cancer

Rho GTPases are important signal transducers in signalling pathways that regulate cell migration, proliferation, survival and death. All these cellular processes are crucial for maintenance of normal tissues, but also contribute to cancer progression [128, 129].The development of human tumours is a multistep process that consists in dysregulation of cell

proliferation, resistance to growth suppressors, inhibition of cell death, uncontrolled replication leading to immortalization and activation of angiogenesis, invasion and metastasis [130].

Accumulation of mutations in genes that affect proliferation and survival is one of the key steps for primary tumour formation. Until recently, it was believed that Rho GTPases were rarely mutated in human tumours. However, it was found that proline 29 in Rac1 is mutated in a subset of melanomas, breast tumours and head and neck tumours [131]. RhoA is also mutated in some tumours including diffuse gastric cancer and angioimmunoblastic T cell lymphoma [132-136]. Although these mutations are likely to be important for tumour development, in most cases, Rho GTPases are found to be upregulated or to have their activity increased by changes in the expression of GAPs, GEFs and/or GDIs [137]. Several Rho GTPases are often upregulated in human tumours including RhoA, RhoC, Rac1, Rac2, Rac3, Cdc42, RhoV and RhoF [137, 138].

The classical Rho GTPases, RhoA, Rac1 and Cdc42, have been shown to be important for the progression and metastasis of different human tumours including breast cancer, liver cancer, melanoma, testicular cancer and ovarian cancer [139-141].

RhoA appears to be involved in almost all stages of tumour progression. For example, in gastric cancer cell lines, RhoA is found to be hyperactive and its suppression leads to partial inhibition of the proliferation phenotype. This effect involves regulation of cell cycle through G1-S progression. Downregulation of the RhoA target mDia1 in gastric cancer cell lines leads to increase in the expression of cell cycle inhibitors $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ while downregulation of ROCK increases the levels of another family of cell cycle inhibitors called INK4 [142]. Several studies *in vitro* and *in vivo* suggest that RhoA has a role during tumour angiogenesis. One example is the knockout of $G\alpha_{13}$ receptors in endothelial cells in mice, which causes a decrease in tumour growth and normalization of tumour vasculature. $G\alpha_{13}$ induces expression of VEGF2 mediated by activation of RhoA which leads to transcription of NFκB [143]. Local invasion is an early step in the metastatic process by which cells need to detach from the primary tumour and migrate through the surrounding tissue. Although RhoA seems to play an active role in cancer progression, recent findings in angioimmunoblastic T cell lymphoma have shown that mutations in RhoA that lead to loss of GTPase activity are important to drive cancer progression [144].

RhoC has also been found to promote tumour progression while RhoB seems to act as a tumour suppressor [145, 146]. RhoC promotes tumour progression in some tumours including melanoma, ovarian cancer and head and neck cancer [147-149]. RhoC is able to control invadopodium formation in tumour cells by locally regulating cofilin activity. Invadopodia are cell protrusions capable of penetrating and degrading the extracellular matrix, increasing the metastatic potential of tumour cells. RhoC activity is spatially regulated by activation of

p190RhoGEF outside the invadopodia and activation of p190RhoGAP inside the invadopodium core. This leads to restriction of cofilin activity only inside the invadopodium core [150]. RhoB, on the other hand, is often downregulated in human tumours [2, 151]. For example, the expression of RhoB in human lung tissues decreases going from normal tissue to invasive carcinoma. In addition, overexpression of RhoB in the lung cancer cell line A549 leads to inhibition of cell growth *in vitro* and *in vivo* [152]. Also, overexpression of RhoB in gastric cancer cell lines inhibits proliferation, migration and invasion; and increases chemosensitivity while overexpression of RhoA and RhoC leads to opposite effects [153].

Rac1 is another Rho GTPase that is found altered in several stages of tumour progression. Deregulation of Rac signalling can be caused by changes in the upstream signalling including tyrosine kinase receptors, PI3Ks, GEFs and GAPs. One example is the activation of Rac1 by Rac GEF P-Rex1 after stimulation of tyrosine kinase receptors and GPCRs in breast cancer cells [154]. In colorectal tumours, the expression of a Rac1 splice variant, Rac1b, is increased in different stages of tumour progression [155]. Expression of Rac1b has also been found in breast cancer and lung cancer [156, 157]. Rac1b has been shown to be involved in MMP-3-mediated malignant transformation of mammary epithelial cells. MMP-3 induces the expression of Rac1b which increases the levels of cellular ROS, leading to expression of the transcription factor Snail, which induces epithelial-mesenchymal transition (EMT) as well as oxidative damage to DNA and genomic instability [158].

The role of Cdc42 in tumour progression may be tissue-specific. Cdc42 is found to be upregulated in many tumours including non-small cell lung cancer, colorectal adenocarcinoma, melanoma, breast cancer and testicular cancer. However, loss of Cdc42 in liver cancer leads to an increase in tumour development [2, 159]. Some other Rho GTPases also appear to have a dual role in tumorigenesis. Rnd3 is downregulated in some tumours and acts as a tumour suppressor [160-162]. However, in some cases, Rnd3 has been shown to be upregulated and mediate drug resistance in cancer cells [163, 164].

RhoBTB1 and RhoBTB2 have been described as tumour suppressors. RhoBTB2 is also known as ‘deleted in breast cancer 2’ (DBC2) and its levels are reduced in some breast cancers [165]. RhoBTB1 is downregulated in some cancers like head and neck cancer [166], and breast cancer and kidney cancer [122]. The *RhoBTB2* gene has been shown to be a target of E2F1, a transcription factor involved in cell cycle and apoptosis [126] and *RhoBTB1* is a target of microRNA-31 (miR-31) in human colon cancer [54].

These examples illustrate how complex the role of Rho GTPases in tumour progression is. Their contributions are dependent on cell type, extracellular stimuli and signalling pathways involved in the particular cancer type or cancer cell line.

Conclusions and future perspective

The classical Rho GTPases, RhoA, Rac1 and Cdc42, are well studied biochemically and for their roles in cancer progression. However, much less is known of the properties of most other family members, which do not appear to be mutated significantly in human cancers. The expression and activity of several family members has, however, been shown to be changed frequently in cancer. RhoC is perhaps the best studied Rho family member in cancer invasion and metastasis [146]. Whether all Rho family members contribute to cancer progression, or have other unique physiological functions to distinguish them from the well-studied subset of Rho proteins, remains to be discovered.

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Figure legends

Figure 1. Signalling pathways regulated by RhoA and Rac1

Examples of signalling by RhoA and Rac1 to the actin cytoskeleton are shown. Different types of cell surface receptors, such as integrins, G-protein-coupled receptors (GPCRs), and tyrosine kinase receptors, activate guanine nucleotide exchange factors (GEFs) for RhoA and Rac1. RhoA and Rac1 then interact with and activate a variety of effectors that are involved in actin polymerization, focal adhesion and stress fibre formation, including protein kinases (e.g. ROCKs, PAKs) and proteins that stimulate actin polymerization (e.g. mDia formins, the WAVE regulatory complex that stimulates the Arp2/3 complex).

Figure 2. The human Rho GTPase family

The Rho GTPase family consists of 20 genes in humans, which are present in all mammals. The family is subdivided into 8 subfamilies: Rac/RhoG subfamily, Rho subfamily, Cdc42/RhoQ/RhoJ subfamily, RhoF/RhoD subfamily, Rnd subfamily, RhoBTB subfamily, RhoH subfamily and RhoU/RhoV subfamily. These subfamilies can be classified as typical (orange circles) or atypical (purple circles) depending on the mode of regulation: typical proteins are regulated by GTP/GDP cycling, whereas atypical proteins have amino acid differences from typical proteins that alter their interactions with GTP and/or GDP. The unrooted phylogenetic tree is based on a ClustlW alignment of the amino-acid sequences of the Rho domains of 20 human Rho GTPase proteins. This does not include splice variants of Rac1 or Cdc42. The figure is modified from [5].

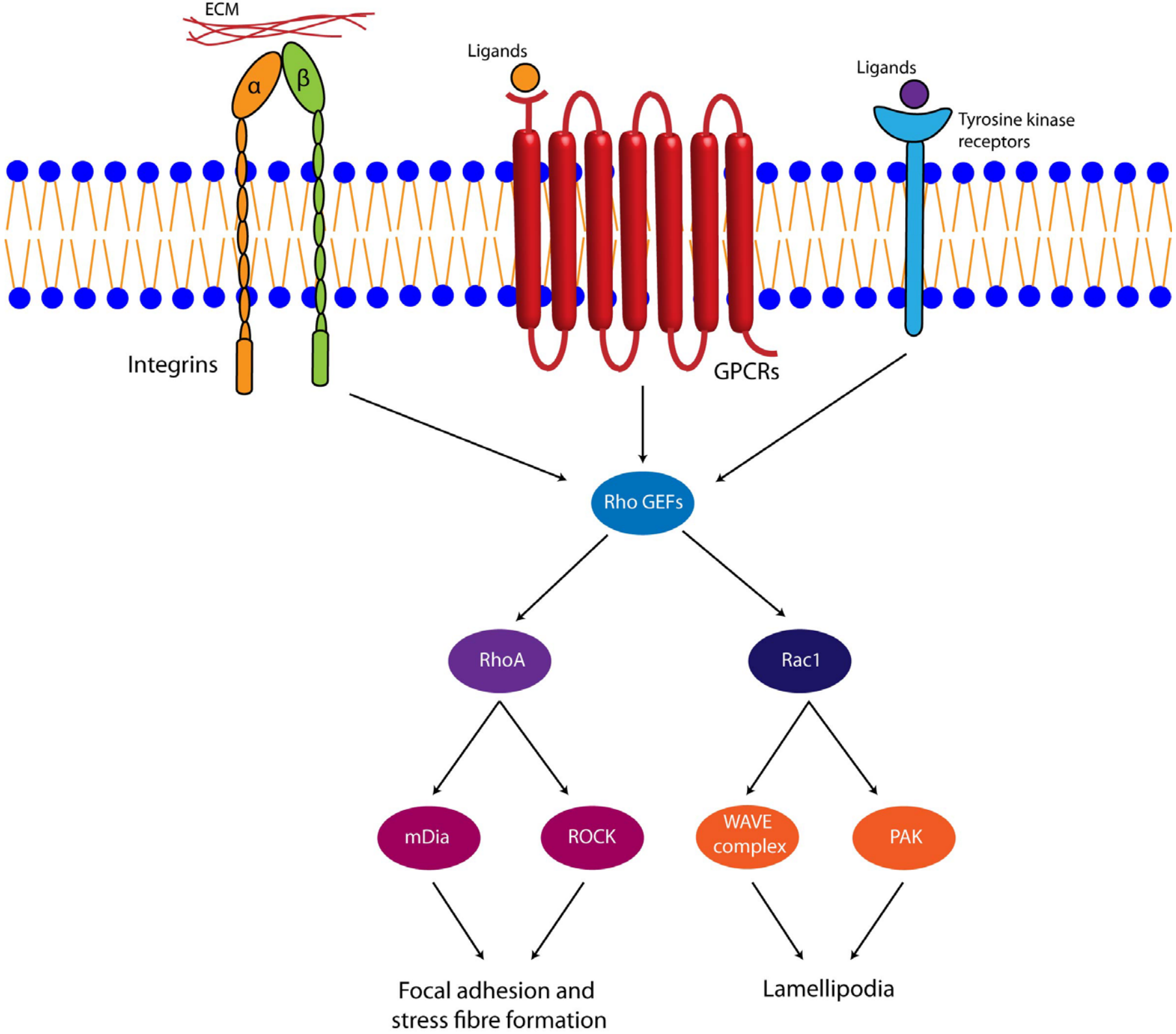
Figure 3. Regulation of Rho GTPases

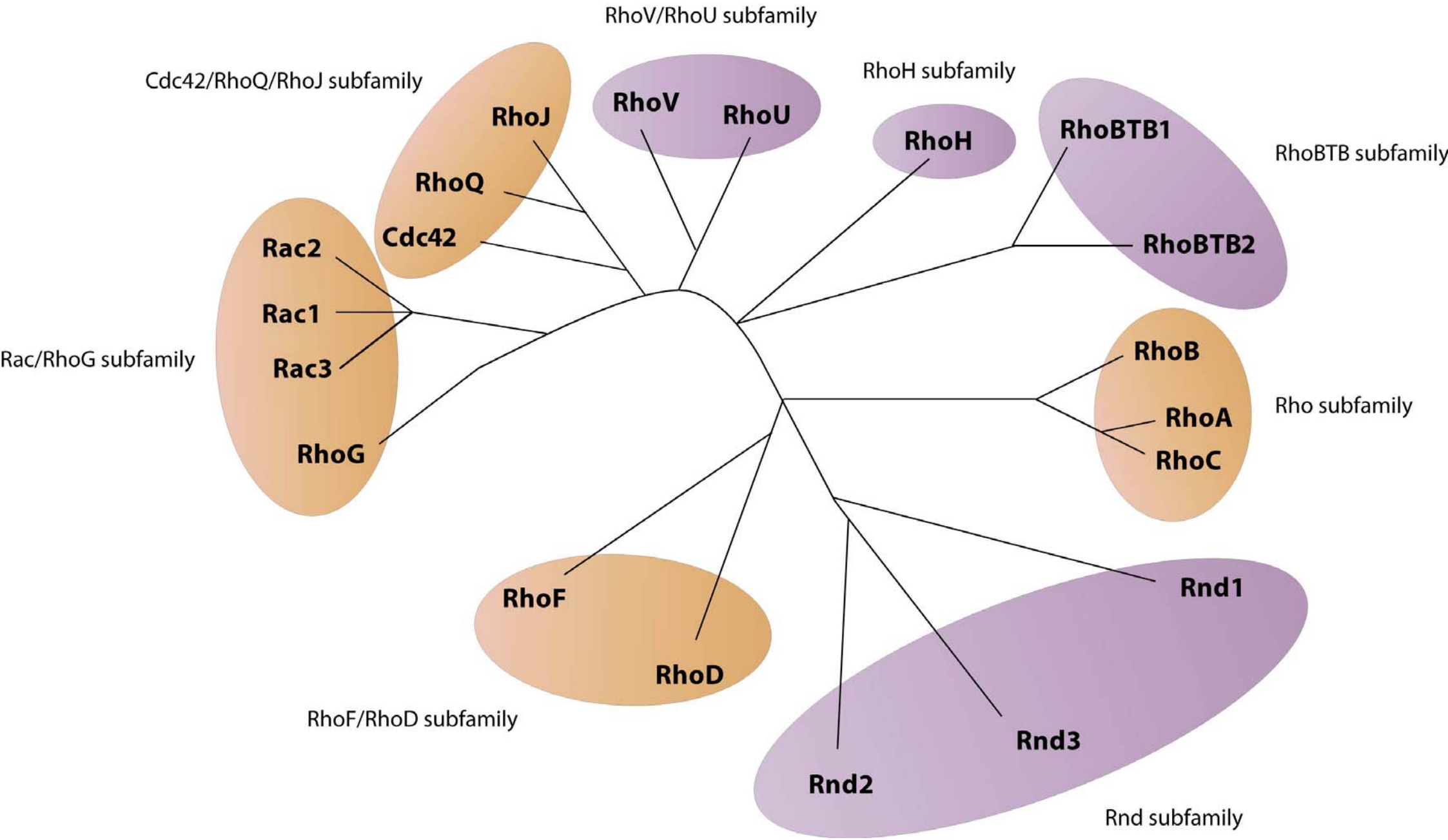
Most Rho GTPases are regulated by GEFs, GAPs and GDIs. These proteins control the cycling between the active GTP-bound form and the inactive GDP-bound form. RhoGDIs can also regulate the localisation and degradation of Rho GTPases. The expression of Rho GTPases are regulated at transcriptional and post-transcriptional (miRNA) levels. Rho GTPases are also be regulated by post-translational modifications (lipid modification, phosphorylation, ubiquitination and SUMOylation), which alter their intracellular localisation, their stability and/or their ability to signal to downstream effectors.

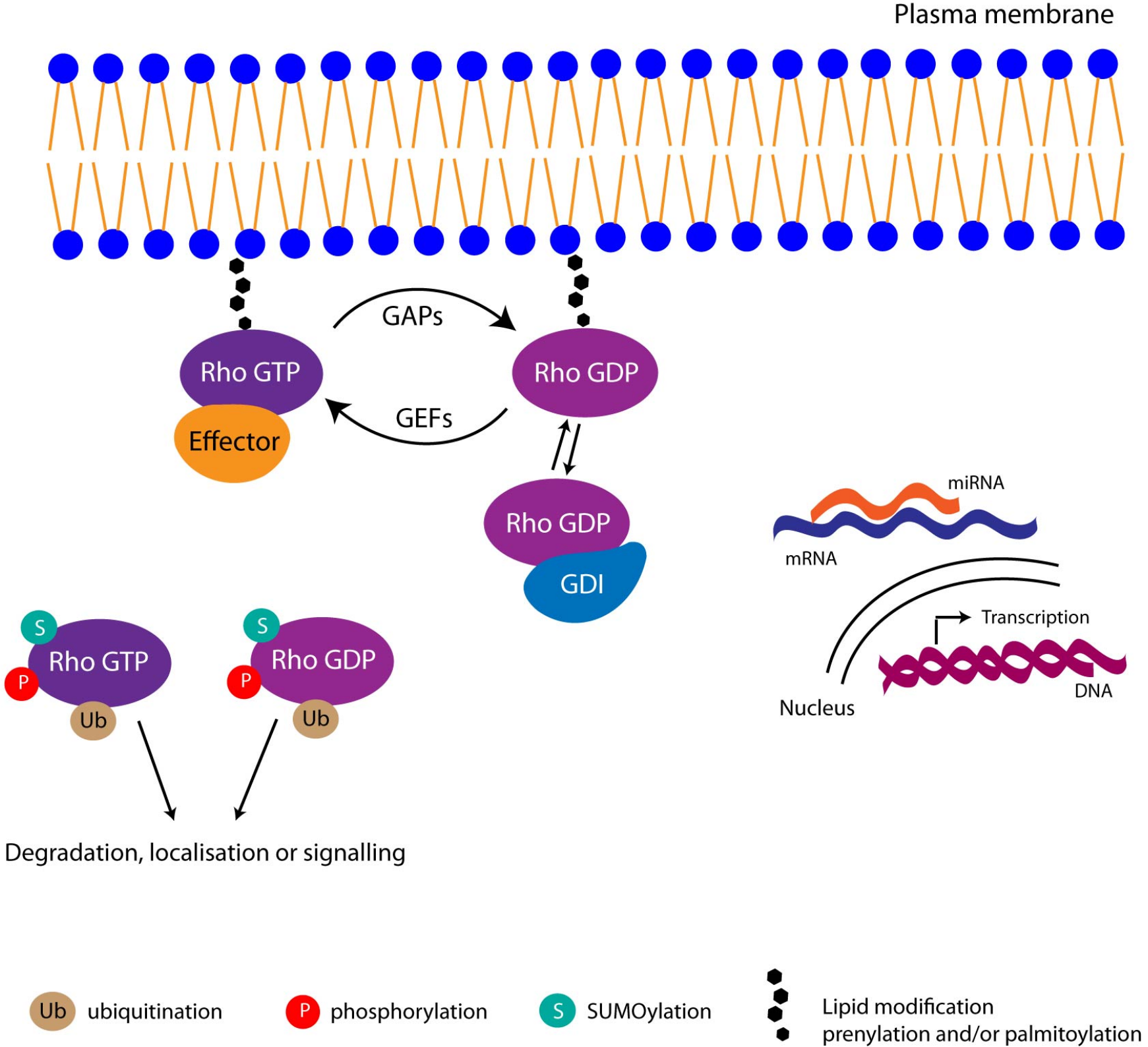
Figure 4. Roles of Rho GTPases in single cell migration

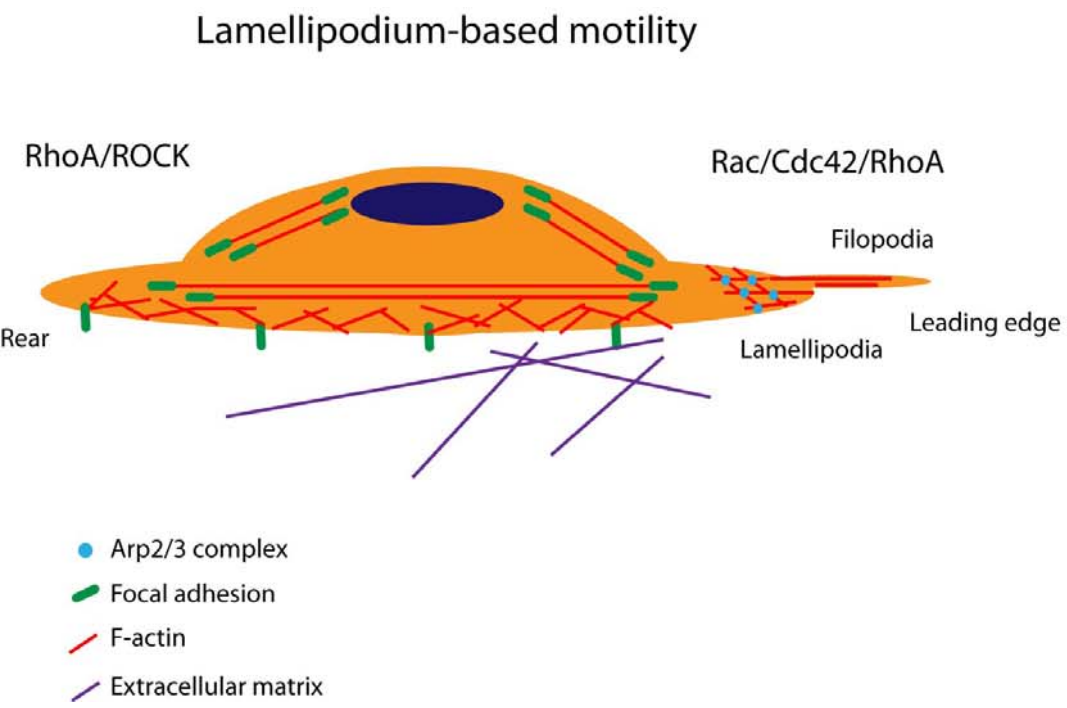
Single cell migration is a multistep process in which cells extend membrane protrusions at the cell front (lamellipodia, filopodia and/or membrane blebs), form new adhesions, contract the cell body and detach the cell rear from the surrounding environment. In lamellipodium-based migration (left), lamellipodium and filopodium formation involves new actin

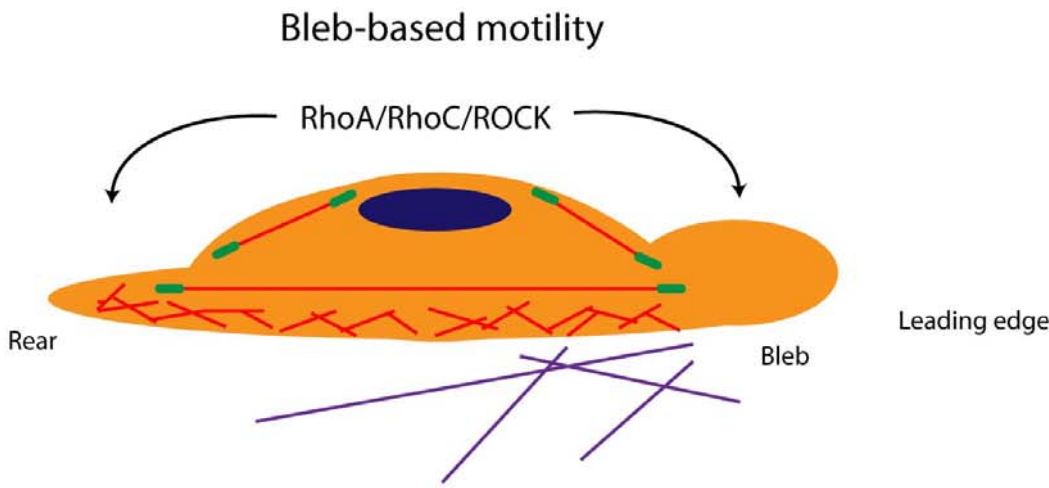
polymerization, and requires actin nucleators such as the Arp2/3 complex that are activated by Rac and Cdc42. Actin polymerization in lamellipodia can also be mediated by formins after RhoA activation. Formation of integrin-based focal adhesions by Rho/ROCK signalling stabilizes lamellipodia and mediates interaction with the extracellular matrix. Actomyosin-mediated contractility and detachment of the cell rear are controlled by Rho/ROCK signalling. In bleb-based migration (right), actomyosin contractility driven by RhoA and/or RhoC activation of ROCKs leads to the extension of blebs, which are protrusions of the cell membrane caused by actomyosin contraction of the cell cortex. Bleb-based migration is less dependent on integrin-mediated adhesion than lamelllipodium-based migration.


ECM
α
β
Integrins
Ligands
GPCRs
Ligands
Tyrosine kinase
receptors
Rho GEFs
RhoA
Rac1
mDia
ROCK
WAVE
complex
PAK
Focal adhesion and
stress fibre formation
Lamellipodia


RhoV/RhoU subfamily
Cdc42/RhoQ/RhoJ subfamily
RhoH subfamily
RhoJ
RhoV
RhoU
RhoH
RhoBTB1
RhoBTB subfamily
RhoQ
Cdc42
RhoBTB2
Rac2
Rac1
Rac3
Rac/RhoG subfamily
RhoB
Rho subfamily
RhoA
RhoC
RhoG
RhoF
Rnd1
RhoD
Rnd3
Rnd2
RhoF/RhoD subfamily
Rnd subfamily


Plasma membrane
GAPs
GEFs
Rho GTP
Effector
Rho GDP
Rho GDP
GDI
miRNA
mRNA
Transcription
DNA
Nucleus
S
P
Rho GTP
Ub
S
P
Rho GDP
Ub
Degradation, localisation or signalling
Ub ubiquitination
P phosphorylation
S SUMOylation
Lipid modification
prenylation and/or palmitoylation


Lamellipodium-based motility
RhoA/ROCK
Rac/Cdc42/RhoA
Filopodia
Rear
Leading edge
Lamellipodia
Arp2/3 complex
Focal adhesion
F-actin
Extracellular matrix


Bleb-based motility
RhoA/RhoC/ROCK
Rear
Bleb
Leading edge